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The developmental expression of the gene for TFIIIA in *Xenopus laevis*

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ABSTRACT

The sequence of the trans-acting positive transcription factor TFIIIA has been deduced by sequencing cDNA and genomic DNA clones. Using DNA from a homozygous diploid animal we show that there is one gene for TFIIIA per haploid genome of *X. laevis*. Protein sequencing of proteolytic fragments of TFIIIA orients the protein in its interaction with the internal control region (ICR) of the 5S RNA gene. The protein lies along the DNA with its carboxyl terminus at the 5' end of the ICR and its amino terminus at the 3' end. The developmental pattern of TFIIIA and its mRNA during oogenesis and embryogenesis are consistent with the idea that the abundance of TFIIIA plays an essential role in the developmental change in 5S RNA gene expression. The change to almost exclusive somatic 5S RNA gene expression by gastrulation occurs using either the TFIIIA that was synthesized by oocyte and/or TFIIIA synthesized from maternal mRNA.

INTRODUCTION

The protein TFIIIA acts as both a positive transcription factor for 5S RNA genes (1) and a specific RNA binding protein that complexes with 5S RNA in *Xenopus* oocytes to form the 7S ribonucleoprotein storage particle (2-3). We describe here the isolation of cDNA and genomic clones of TFIIIA and their use in the study of the structure and developmental expression of the protein and its gene. In the course of this work, the sequence of a full-length cDNA clone of TFIIIA mRNA was published by Ginsberg *et al.* (4).

The sequence that we found for TFIIIA from cDNA and genomic clones is identical to the one already published (4) except for a single nucleotide (and amino acid) difference. This report adds to the information already published (4) by demonstrating that there is just a single gene for TFIIIA in the *X. laevis* genome and that the protein is oriented along the 5S RNA gene with its carboxyl terminus toward the 5' end and its amino terminus at the 3' end of the internal control region.

MATERIALS AND METHODS**Protein Purification and Sequencing.**

TFIIIA was prepared from 7S ribonucleoprotein particles by the procedure of Smith *et al.* (5). TFIIIA was digested with cyanogen bromide as described

(5) and the largest cyanogen bromide peptide (20 kd) was purified from polyacrylamide gels and sequenced by gas phase microsequencing (6a, 6b). The 20 and 30 kd proteolytic fragments of TFIIIA were prepared by digestion of the 7S particle (5) as described previously.

Preparation of RNAs.

Total RNA was isolated from immature ovaries obtained from female Xenopus laevis from 2.5-5cm in length (7). Two g of tissue were homogenized in 60 ml of 4M guanidinium thiocyanate, 1% N-lauryl sarcosine, 50 mM EDTA, and 5 mM sodium citrate pH 7.0 with 0.1% antifoam A (Sigma) and 0.1 M β -mercaptoethanol added prior to homogenization. The homogenate was then centrifuged at 8,000 X g for 10 min at 10° and the supernatant fluid collected. This material was divided into six aliquots and layered onto 1.4 ml of 5.7 M CsCl₂ pH 7.0, 1 mM EDTA and centrifuged at 164,000 X g at 20° for 17 h in a Beckman SW41 rotor (35,000 RPM). The RNA pellets from 6 SW41 tubes were then resuspended in 3 ml total volume of guanidine thiocyanate solution without antifoam A or β -mercaptoethanol and centrifuged at 8,000 X g. The RNA was precipitated from the supernatant liquid by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol. Total RNA was collected by centrifugation at 8,000 X g and the pellet rinsed with 70% ethanol and then dried in vacuo. The pellet was resuspended in 10 mM Tris pH 7.5, 1 mM EDTA with 0.1% SDS and the RNA concentration determined by absorbance at 260 nm. Polyadenylated RNA was purified by two rounds of affinity chromatography on oligo dT cellulose (8). Polyadenylated RNA from 4 ml of packed Xenopus laevis tissue culture cells (derived from kidney) was prepared in the same fashion. Total RNA was prepared from 100 oocytes defolliculated with collagenase and/or dejellied embryos of X. laevis as described above.

Synthesis and Construction of cDNA Libraries.

Double stranded cDNAs were prepared from 100 μ g of polyadenylated immature ovary RNA essentially as described (9, 10). After the double stranded cDNA was prepared, blunt ends were generated by treatment with S1 nuclease and the Klenow fragment of DNA polymerase I (11). Phosphorylated Bam HI or Hind III linkers were ligated to this material and the linkers were then trimmed with the appropriate restriction enzyme. This material was extracted with phenol and fractionated on a 1.0 X 30 cm column of Sepahrose CL-4B and the excluded fractions collected and ethanol precipitated (11). This material (approximately 3 μ g of DNA determined by incorporation of radioactive dCTP) was ligated to 10 μ g of pUC 9 that had been digested with the appropriate restriction enzyme and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). The recombinant plasmids thus generated were

transformed in the *E. coli* strain DH1 (12). Transformed bacteria were plated on L-agar plates containing 150 µg/ml ampicillin.

Screening of cDNA Libraries for Plasmids Containing TFIIIA cDNA Sequence.

After the recombinant plasmids were immobilized on nitrocellulose (13), the filters were prewashed in 2 X SSPE (20 X SSPE is 0.2 M NaH₂PO₄, 3 M NaCl, and 20 mM EDTA) with 0.5% prehybridized in 5 X SSPE and 5 X Denhardt's solution (50 X Denhardt's solution is 1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA) for 6 h at 37°. To identify bacterial colonies harboring plasmids corresponding to TFIIIA mRNA these filters were hybridized for 24 h at 37° in the same solution used for prehybridization with 1.25 X 10⁵ cpm/ml of an end-labelled mixture of 32 possible 5'-labeled 19 nucleotide probes (Fig. 1B), the sequence of which was derived from the protein sequence near the amino terminus of a cyanogen bromide fragment of TFIIIA (Fig. 1A). The filters were washed at room temperature twice in 3 X SSPE with 0.5% SDS and once at 42° in 3 X SSPE with 0.1% SDS for 30 min. Bacterial colonies which hybridized to the labeled oligonucleotides were subjected to secondary and tertiary screening.

Genomic Clones of TFIIIA.

Genomic DNA from a homozygous diploid *Xenopus laevis* was digested with a fivefold excess of Bam HI according to the manufacturer's instructions. This material was then applied to a potassium acetate gradient (5%-20%) and centrifuged as described (11). The DNA fragments corresponding to 9 kb and above were collected and precipitated. Phage lambda arms from Charon 35 were prepared in a similar fashion after digestion with a fivefold excess of Bam HI. The object was to isolate a Bam HI fragment that we knew hybridized with the most 5' part of the CNDA clone (Figure 2A). One µg of the digested homozygous diploid DNA was ligated to 3 µg of Charon 35 arms overnight at 15° (11). This material was then packed in vitro (Packagene Vector Cloning Systems) and plated on ten 150 mm plates. Nitrocellulose replicas of these plates were made (11) and screened with a 5' specific RNA probe derived from the TFIIIA cDNA clone. Plaques which hybridized were then subjected to secondary and tertiary screening as described (11).

Electrophoresis and Blotting of Nucleic Acids.

Ten µg of genomic DNA from individual *Xenopus laevis* was digested to completion with various restriction enzymes, electrophoresed through 0.7% agarose gels and the DNA transferred to nitrocellulose (11).

RNA was isolated from various *Xenopus laevis* tissues as described above. RNAs were electrophoresed in 1.2% agarose gels containing 20 mM methyl mercuric hydroxide (14), soaked for 30 min in 0.5 M ammonium acetate and blotted to nitrocellulose overnight (15). RNA immobilized to nitrocellulose was hybridized to the TFIIIA RNA probes as described below.

Radioactive RNA probes were derived from transcription of cDNA clones of the insert of pTF1 (see text for description of pTF1) inserted into the vector pSP64 (16) and then linearized with EcoRI. The 3' specific RNA probe was transcribed from the plasmid pTF1 insert that had been linearized with Hinf I. This 3' specific probe is delimited by a Hinf I site at nucleotide 999 and the Hind III linker (nucleotide 1120) beyond the translation termination codon. Nucleotide numbering refers to the full-length published cDNA sequence (4). A 5' specific probe was also used but the data is not shown. It encompassed the Bam linker at the 5' end of pTF1 (nucleotide 85) to a Bgl II site (nucleotide 343). Hybridizations of these probes to the Southern and Northern blots were performed after the blots were first prehybridized in 4 X SSPE, 5X Denhardt's solution, 100 µg/ml of denatured calf thymus DNA, and 500 µg/ml yeast RNA (Sigma) for at least 4 h at 42°. Hybridization were performed in prehybridization solution with the addition of 10% dextran sulfate (Sigma) at 42° for 12-16 h. After hybridization, the blots were washed with 50% formamide, 4 X SSPE, and 0.5% SDS twice for 30 min each at 42° and twice for 30 min in 0.1 X SSPE with 0.1% SDS at 65°.

RESULTS

Amino Acid Sequencing of TFIIIA Peptides and Synthesis of an Oligonucleotide Probe.

Attempts to sequence intact TFIIIA failed because its N-terminus is blocked. The chemical nature of this blocked N-terminus is unknown. We, therefore, digested TFIIIA with cyanogen bromide which has been demonstrated previously to produce an internal 20 kd polypeptide flanked by two fragments of approximately 10 kd (5). The 20 kd polypeptide was purified by SDS polyacrylamide gel electrophoresis, eluted from the gel and subjected to gas phase microsequencing (6a, 6b). The first 26 amino acids of this sequence are shown in figure 1A. The sequence from position 18-24 (Cys-His-Phe-Glu-Asn-Cys-Gly) was chosen to prepare a mixed oligonucleotide probe complementary to the predicted mRNA sequence (figure 1B).

Preparation and Screening of a Xenopus Ovary cDNA Library and Isolation and Sequencing of TFIIIA Clones.

The mixed oligonucleotide probe was labeled at the 5' end with polynucleotide kinase and gamma (³²P)-ATP and used to screen cDNA libraries prepared from poly(A)+ RNA isolated from immature *X. laevis* ovaries. A single bacterial colony that hybridized to the oligonucleotide probes was identified after screening approximately 5000 colonies from one library. This colony harbored a plasmid (pTF1), with an approximately 1000 base pair insert. This insert was sequenced in both directions as single stranded DNA containing

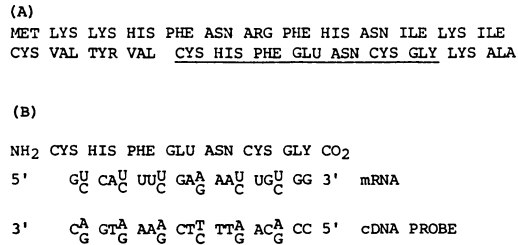


Fig. 1. Amino acid sequence at the amino terminus of the 20 kd cyanogen bromide fragment of TFIIIA. The 20 kd large fragment from the cyanogen bromide digest of TFIIIA was subjected to gas phase microsequencing; A) the sequence of the first 28 amino acids. The methionine at position one does not actually appear in the sequence but is inferred from the cyanogen bromide digest. The underlined amino acids (18-24) were chosen as the basis of synthesis of a mixed oligonucleotide probe (B).

fragments of the cDNA subcloned into ml3, mp8, and 9 using standard dideoxy methodology. The nucleotide sequence of the clone and the predicted amino sequence of the encoded protein was analyzed. The putative protein should have two internal methionines which, when cleaved by cyanogen bromide, would yield the expected single internal 20 kd and two external 10 kd polypeptides. Near the N-terminus of the internal polypeptide is the exact amino acid sequence obtained by microsequencing the larger internal cyanogen bromide fragment. This cDNA clone ends just after the translation termination codon and contains all but 28 amino acids at the N-terminus. A second cDNA clone, pTF6, was isolated from another cDNA library; it contains all but 15 amino acids of the N-terminus of TFIIIA. The remainder of the 5' end of the sequence was obtained from a genomic clone of TFIIIA.

Determination of the Orientation of the 20 kd and 30 kd Fragments of TFIIIA.

Smith *et al.* (5) reported that limited proteolysis of TFIIIA with papain or trypsin produces a 30 kd and a 20 kd polypeptide, respectively. The location of the cleavage sites was not determined. Sequencing showed that the 30 kd fragment like intact TFIIIA, has a blocked amino-terminus. However, an N-terminal sequence was obtained for the 20 kd fragment that begins at amino acid 5 from the N-terminus of intact TFIIIA. This indicates that the 30 kd fragment is generated following the loss of amino acids from the carboxyl terminus; the 20 kd polypeptide results from proteolysis primarily of the carboxyl end with the additional loss of four amino acids at the N-terminus. Thus, the binding domain of TFIIIA consists of the amino terminal 30 kd region of the protein oriented along the DNA with the protein's amino terminus at the 3' end of the internal control region (ICR) of the 5S RNA gene.

Southern Blot Analysis of the TFIIIA Gene of *Xenopus laevis*.

The only difference between our sequence and that published by Ginsberg *et*

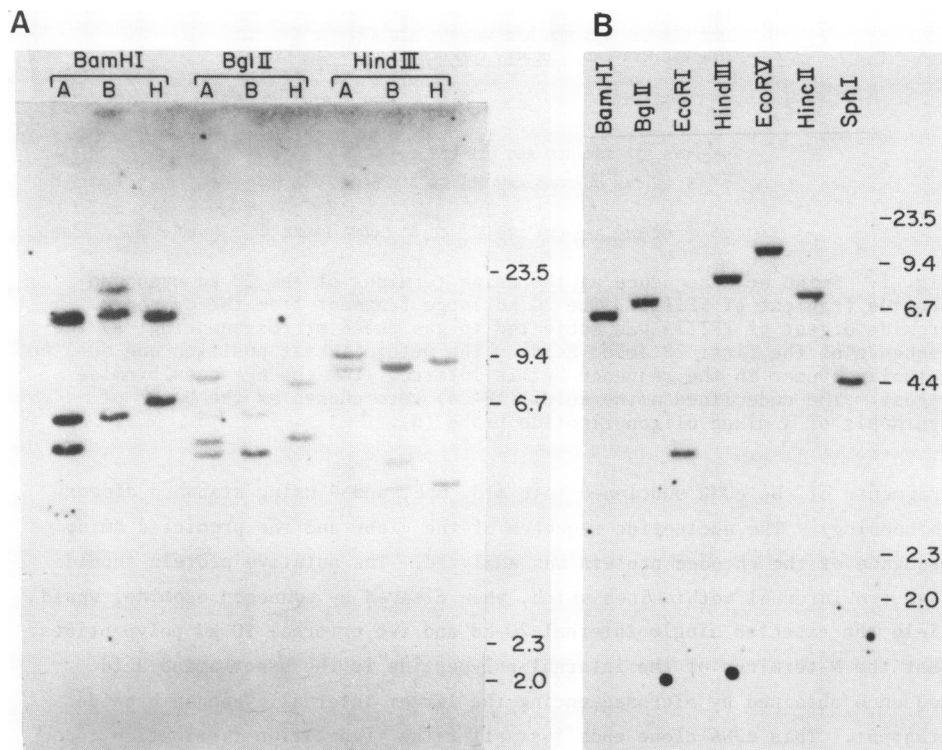


Fig. 2. Detection of TFIIIA gene by hybridization to *X. laevis* genomic DNA. (A) Genomic DNA from 2 adult *Xenopus laevis* imported from South Africa (lanes labeled A and B) and one homozygous diploid *Xenopus laevis* (H) were digested with the designated restriction enzymes, electrophoresed on a 0.7% agarose gel and the gel then blotted to nitrocellulose. The transferred DNA was hybridized to single stranded RNA probes from the complete cDNA probe for TFIIIA. Size markers are shown on the right.

(B) Genomic blots of homozygous diploid *Xenopus laevis* DNA hybridized to a 3' specific probe (see Materials and Methods).

al. (4) is the presence of an adenine residue at nucleotide 220 (counting the A residue of the first AUG as number one) instead of a cytosine as they reported. This is presumed to be due to polymorphism and results in the occurrence of a lysine in our cDNA at amino acid number 74 where a threonine exists in theirs. Polymorphism in the *Xenopus* genome for the TFIIIA gene is readily seen from a genomic DNA blot (figure 2A). Heterogeneity was detected in DNA preparations from different individuals for a variety of restriction enzyme digests. To show that polymorphism could explain this heterogeneity we analyzed genomic DNA from a single homozygous diploid *Xenopus laevis* (17) (lanes labeled H). It is apparent from these comparisons that the homozygous

diploid frog has a less complex pattern for each restriction digest than that of normal Xenopus laevis. We tested the possibility that a single gene encodes TFIIIA by hybridizing blots of DNA from the homozygous diploid Xenopus with a probe corresponding to the 3' end of the TFIIIA cDNA clone (Fig. 2B). A variety of restriction enzymes yield just one band that hybridizes with the 3' specific probe, confirming the fact that the X. laevis genome contains a single gene for TFIIIA (Fig. 2B). The same result was found with a 5' specific probe (described in Methods; data not shown).

Transcripts and Developmental Control of the TFIIIA Gene.

TFIIIA protein levels follow the transcriptional activity of oocyte type 5S RNA genes during embryogenesis (18, 19). TFIIIA is present in very high levels in maturing oocytes, when oocyte type 5S genes are being expressed, and present in much lower levels in somatic cells where the oocyte type genes are not expressed (18, 19). Increasing the amount of TFIIIA in embryos elevates oocyte 5S RNA gene transcription (20). Somatic cells contain a protein that cross-reacts with antibody to TFIIIA but migrates more slowly on SDS polyacrylamide gels than does the TFIIIA from oocytes (18, 19). These various facts have led us to measure the amounts and size of TFIIIA mRNA at various stages of oogenesis, embryogenesis and in somatic cells. TFIIIA mRNA levels were measured by isolation of total RNA from oocytes, early embryos, and somatic tissue culture cells, then electrophoretically separating these RNAs in agarose gels containing methylmercuric hydroxide. The RNA was transferred to nitrocellulose and hybridized to labeled RNA probes corresponding to the complementary strand of TFIIIA mRNA. In Xenopus oocytes, a single band corresponding in size to approximately 1600 nucleotides hybridizes to the probe for TFIIIA, while a broad band of a slightly larger molecular weight from somatic cells hybridizes to the same probe (figure 4A).

The amount of the mRNA for TFIIIA at various stages roughly correlates with published levels of TFIIIA (18, 19). The parallel, however, is not exact. For example, the TFIIIA level is reported to drop 10-20 fold (18, 19) during ovulation (meiosis) while TFIIIA mRNA levels remain unchanged (Fig. 3B). During early cleavage stages of embryogenesis, approximately stages 1-8 (21), TFIIIA mRNA levels remain constant per embryo (figure 3C and data not shown); then during gastrulation sometime after stage 8, before stage 12, TFIIIA mRNA concentrations drop to barely detectable levels. This drop in the mRNA of TFIIIA was observed also by Ginsberg *et al.* (4).

We have determined that TFIIIA mRNA levels range from about 20 million copies in a stage 1 oocyte to about 6 copies of TFIIIA mRNA per somatic tissue culture cell. This is in agreement with the levels of TFIIIA mRNA reported

previously (4). The mRNA gradually increases in the swimming embryo (between stages 25 and 32). The newly synthesized "somatic" mRNA migrates as a broad smear that is slightly larger than the ovarian mRNA for TFIIIA.

DISCUSSION

This study in part duplicates that of Ginsberg *et al.* (4), who have published the complete sequence of a full-length TFIIIA cDNA clone. They also described similar mRNA quantitations the most interesting feature of which is the loss of inherited TFIIIA mRNA during gastrulation. This abrupt loss of TFIIIA mRNA is not accompanied by a loss of TFIIIA itself and occurs just at the onset of transcription in *Xenopus* embryos; it helps to explain why the transcription of 5S RNA genes starts at such a very low level in embryos (22,

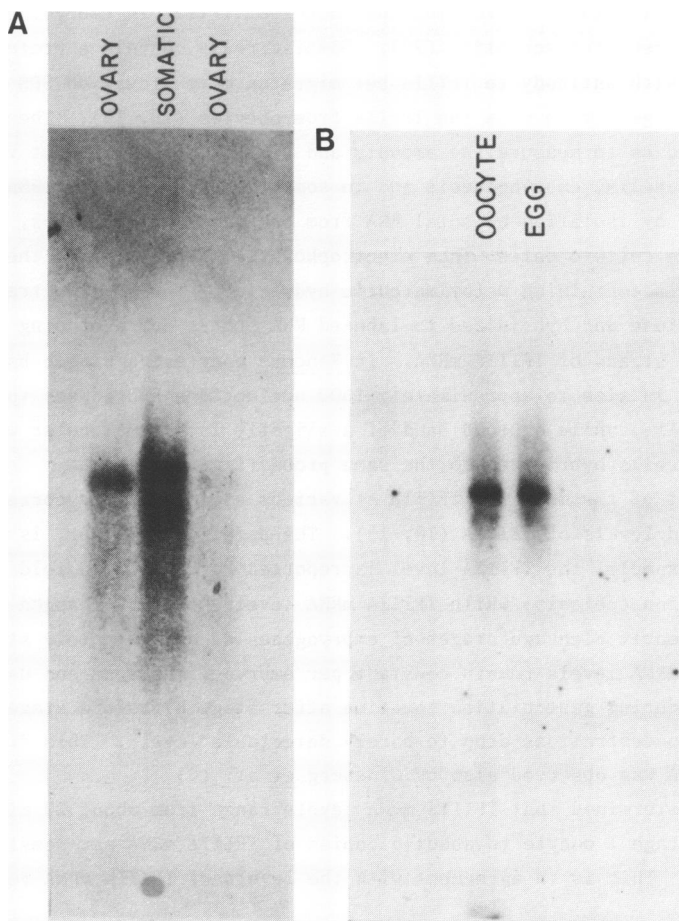




Fig. 3. Northern blot analysis of poly(A)⁺-RNA from immature *Xenopus laevis* ovaries and somatic tissue culture cells (A) Poly(A)⁺-RNA from immature ovaries or somatic tissue culture cells from *Xenopus laevis* was isolated and then electrophoresed on a 1.2% agarose gel containing 10 mM methyl mercury hydroxide. This gel was then blotted to nitrocellulose and hybridized to a single stranded RNA probe corresponding to the entire TFIIIA cDNA. The lanes are from left to right 50 ng immature ovary poly(A)⁺-RNA, 3 μ g of poly(A)⁺-RNA from cultured cells, and 10 ng of immature ovary poly(A)⁺-RNA.

(B) Northern blot analysis of RNA from *Xenopus laevis* stage 6 oocytes and the same number of unfertilized eggs prepared from the same female.

(C) Northern blot analysis of RNAs from various stages of embryogenesis.

Total RNA from the indicated stages of embryogenesis was isolated and the RNA from approximately 5 embryos electrophoresed through an agarose gel containing 10 mM methyl mercuric hydroxide blotted and transferred RNAs hybridized with a single stranded RNA probe. Lanes from left to right 50 ng immature ovary poly(A)⁺-RNA, stages 8, 12, 25 embryo RNA, 10 ng immature ovary poly(A)⁺-RNA, stages 32, 39 and 41 embryo RNA, and 50 ng immature ovary poly A RNA. Embryonic stages from Nieuwkoop and Faber (21).

23). It is with the use of TFIIIA synthesized by oocytes that the embryo effects the transition from predominantly oocytes-type 5S RNA synthesis in oocytes to about equal oocyte and somatic 5S RNA synthesis at blastulation (BMT) and finally to somatic 5S RNA gene expression exclusively at gastrulation and thereafter (22, 23).

Just a single gene encodes TFIIIA in the *X. laevis* genome. This was proven by analyzing DNA from a homozygous diploid individual *Xenopus* kindly provided to us by D. Reinschmidt and R. Tompkins (17). Thus, the different sized somatic TFIIIA which is first detectable in tailbud stage embryos is not likely to be the product of a separate gene. The size of TFIIIA mRNA from somatic cells is slightly larger than that from oocytes (Fig. 3A) (4), but the size difference may be due to the length of poly A tracts. Whatever the explanation for a differently migrating TFIIIA-related protein, it appears that the transition from oocyte-type 5S RNA gene expression to almost exclusively somatic-type 5S RNA synthesis in somatic cells can be effected just by modulation of the level of the type of TFIIIA synthesized in oocytes.

In this paper we have oriented the carboxyl and amino terminal ends of TFIIIA with respect to their orientation along the internal control region of the 5S RNA gene. At the carboxyl end of the protein there is a region that is involved in transcription of the 5S RNA genes presumably by interacting with one of the other factors required to form a transcription complex or with RNA polymerase III. This is followed by a stretch of amino acids that constitute the binding domain.

Recently, Miller *et al.* have suggested a structure for this binding domain based on the observation that the functional protein is complexed with nine to eleven zinc atoms (24). Each zinc atom is thought to be complexed with a domain of about 30 amino acids. This zinc-binding region comprises all but the first 12 amino acids at the amino terminus and the last 68 amino acids at the carboxyl terminus. Recently, sequencing carried out by Tso *et al.* (25) has located all six exon-intron boundaries, three of which delimit the proposed domains of Miller *et al.* This is independent evidence for the repeating structure of this protein.

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